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CERTIFICATE

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I hereby certify that annexed is a true copy of the Provisional Specification as filed on 19 June 2002 with an application for Letters Patent number 519667 made by ALAN MURRAY; CHRISTINE DUPONT; MASSEY UNIVERSITY; INSTITUTE PASTEUR.

Dated 7 July 2003.

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PROVISIONAL SPECIFICATION

PROTEIN

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We, ALAN MURRAY, a New Zealand citizen, of 6 Williams Terrace, Palmerston North, New Zealand, and CHRISTINE DUPONT, a New Zealand citizen, of 5 Luton Street, Palmerston North, New Zealand in trust for MASSEY UNIVERSITY, a body corporate established under the Massey University Act 1963 and Education Amendment Act 1990 of Palmerston North, New Zealand, and INSTITUTE PASTEUR, of 25, rue du Dr. Roux, F-75724 Paris Cedex 15, France, do hereby declare this invention to be described in the following statement:

PROTEIN

This invention relates to a protein and compositions which contain it. More particularly, it relates to an exported protein identified in culture filtrate from *Mycobacterium avium* subspecies *paratuberculosis*.

BACKGROUND

Johne's disease (paratuberculosis) is a chronic wasting disease of ruminant animals caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*M. ptb*).

The disease spreads insidiously, with animals becoming infected early in life following ingestion of contaminated milk, collostrum, or pasture. In many countries, including New Zealand and the USA, herds are not routinely tested for *M. ptb* infection. The number of herds officially known to have infection is therefore thought to be a gross underestimate of the actual level of infection. Estimates suggest that approximately 60% of dairy herds in New Zealand are infected with *M. ptb*. In sheep, prevalence of *M. ptb* infection may be as high as 70%.

Infected animals are less productive, which results in significant economic losses for farmers. The cost to New Zealand farmers is estimated to be in excess of $30 million per annum. The economic impact in the USA was estimated to be as much as US$1.7 billion per annum (Chiodini *et al.*, 1984a).

Good management practices, herd testing, and culling of infected animals are important tools for controlling paratuberculosis. However, this strategy alone is unlikely to completely control the problem because current diagnostic tests frequently fail to identify infected animals in the early, preclinical stages of disease. The early stages of disease are asymptomatic and shedding of the organism in the faeces is undetectable or intermittent. Only animals which progress to the terminal stage generally show clinical disease, and that only after two to five years (Stuart, 1965; Lepper *et al.*, 1989). For this reason, it is

suggested that for every clinical case on a farm, there are approximately 20 additional infected animals.

Detection of infection in the host is influenced by the stage of disease (Ridge *et al.*, 1991; Sweeney *et al.*, 1995). In the lengthy subclinical stage, the bacterium produces little or no detectable immune response and the number of organisms is typically low, making direct detection of the bacterium difficult. In cattle, culture of *M. ptb* from faeces or tissue is currently the most accurate means of detecting infection. The success of culture is linked to the presence of sufficient numbers of *M. ptb* being shed from the intestine. Hence, animals in the early stages of disease, which do not shed the bacterium, or shed intermittently, are difficult to detect by this method. A further disadvantage of culture is the long incubation time required for the appearance of colonies.

Serological testing is also available, however, this performs best in animals with clinical disease (80% detection) and poorly with subclinically infected animals (as low as 15% detection), (Billman-Jacobe *et al.*, 1992; Sweeney *et al.*, 1995).

Alternatively, *M. ptb* can be confirmed through PCR to identify the presence of the species-specific DNA fragments. To date, only three subspecies-specific DNA fragments have been identified in the *M. a. ptb* genome.

Of these, the most widely used is the IS*900* element. IS*900* detection by PCR is available as a commercial kit (Idexx, USA). This test is reported to have a sensitivity of approximately 60% in infected cattle, based on cattle diagnosed by faecal culture (Whipple *et al.*, 1992). A report of finding organisms that give positive results with IS*900* PCR but are not *M. ptb* has placed some doubt on the routine use of this test as the ultimate confirmation of *M. ptb* (Cousins *et al.*, 1999).

Eradication of Johne's disease is difficult due to the inability of current diagnostic tests to detect all infected animals. As a consequence, more animals are put at risk of infection because preclinically infected animals intermittently shed the bacterium, thereby spreading

the organism. Therefore, improvements in the sensitivity of diagnostic testing and/or increased immunity of uninfected animals would be beneficial.

Commercially available vaccines for the control of Johne's disease contain whole organisms, either attenuated strains of *M. ptb* (eg. Neoparasec - Merial, France), or heat-killed preparations (eg. Gudair - CZ Veterinaria, Porrino, Spain), which are mixed with an oily adjuvant and injected subcutaneously. Vaccination does not prevent or eliminate infection, but reduces the number of animals that progress to clinical disease and the excretion of organisms in the faeces.

Unfortunately, these whole cell vaccines come with a number of drawbacks. Firstly, the immunogenic load presented by the whole cell in conjunction with the adjuvant induces a severe hypersensitivity reaction at the injection site, which can cause the formation of a persistent nodule (granuloma). Occasional rupture of the nodule causes suffering to the animal and potential downgrading of the carcass at slaughter, with concomitant diminished returns for the farmer. Histological examination of nodules or regional lymph nodes can reveal the presence of acid-fast organisms that can be confused with the tuberculosis organism. Additionally, while Johne's disease is distinct from tuberculosis, and caused by a distinct organism, the current Johne's vaccines can generate cross-reactive responses to *M. bovis* skin test antigens which can interfere with tuberculosis control programmes.

Accordingly, there is a need for alternative methods for detecting Johne's disease in animals, and proteins and/or markers and/or vaccines useful in such methods.

The applicants have identified and characterized a novel protein, from *M. ptb*, which exhibits strong potential as a diagnostic marker and a subunit vaccine. This protein can be readily obtained from a culture filtrate of *M. ptb*, or expressed in a heterologous host, as the gene encoding this protein has also been identified and characterized by the applicants. It is towards this protein that the present invention is broadly directed.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect the present invention provides a protein which has the amino acid sequence set forth in Figure 1, or a functionally equivalent variant or fragment thereof which has greater than 77% amino acid sequence identity with said protein.

In a preferred embodiment, the protein has the amino acid sequence of amino acids 20 to 235 as set forth in Figure 1, or a functionally equivalent variant or fragment thereof which has greater than 77% amino acid sequence identity with said protein.

As defined herein, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent polypeptide is immunologically cross-reactive.

In one embodiment, said protein is obtainable from *M. ptb*.

In another embodiment, said protein or functionally equivalent variant or fragment is obtainable from a bacterium other than *M. ptb*.

In another embodiment, said protein is obtainable from a heterologous host transformed with a polynucleotide which encodes said protein or functionally equivalent variant or fragment thereof and is capable of expressing said polypeptide.

In another aspect, the invention provides a polynucleotide or fragment or variant thereof comprising a gene sequence as set forth in Figure 2, which encodes a protein of the invention.

In another aspect, the invention provides oligonucleotide or polynucleotide primers capable of amplifying a polynucleotide, which encodes a protein or functionally equivalent variant or fragment thereof as described above, in a Polymerase Chain Reaction.

In a further aspect, the invention provides antibodies or other ligands which bind a protein or functionally equivalent variant or polypeptide fragment of the invention. The antibody may also comprise an antibody binding fragment.

In another aspect, the invention provides a composition which comprises a protein or functionally equivalent variant or fragment as defined above.

In one embodiment, the composition and/or protein may have utility as a diagnostic capable of detecting the presence of *M. ptb.*

In one embodiment, the composition and/or protein may have utility as a diagnostic capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In another embodiment, the composition may be a vaccine composition, comprising a protein or functionally equivalent variant or fragment of the invention together with an acceptable diluent, carrier, excipient, or adjuvant, said polypeptide being present in an amount sufficient to generate a protective immune response to *M. ptb* infection.

In another aspect, the invention provides a composition comprising a polynucleotide which encodes a protein or functionally equivalent variant or fragment thereof as described above.

In yet another aspect, the invention provides a composition comprising oligonucleotide or polynucleotide primers capable of amplifying a polynucleotide which encodes a protein or functionally equivalent variant or fragment thereof as described above in a Polymerase Chain Reaction.

In another aspect, the invention provides a composition comprising an antibody or other ligand that bind a protein of the invention or functionally equivalent variant or fragment thereof as defined above.

In one embodiment, the composition may be a component in an assay.

In another embodiment, the composition may be a component of an assay kit.

In another embodiment, the composition may have utility as a diagnostic capable of detecting the presence of *M. ptb*.

In yet another embodiment, the composition may have utility as a diagnostic capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In another aspect, the invention provides a method of detecting Johne's disease in an animal comprising contacting a sample with a protein of the invention or a composition comprising a protein of the invention and detecting a response indicative of the presence of *M. ptb*.

In one embodiment, the method comprises contacting an animal with a protein of the invention or a composition comprising a protein of the invention and detecting a delayed-type hypersensitivity response.

In another embodiment, the method comprises contacting a sample with a protein of the invention or a composition comprising a protein of the invention and detecting in the sample the presence of antibodies and/or ligands that bind a protein of the invention.

In another embodiment, the method is capable of detecting the presence of *M. ptb*.

In yet another embodiment, the method is capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In another aspect, the invention provides a method of detecting Johne's disease in an animal comprising contacting a sample with antibody and/or ligand that binds a protein of the invention and detecting the presence of said bound antibody and/or ligand.

In one embodiment, the method is capable of detecting the presence of *M. ptb*.

In yet another embodiment, the method is capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In another aspect, the invention provides a method of detecting Johne's disease in an animal comprising contacting a sample with a composition comprising oligonucleotide or polynucleotide primers capable of amplifying a polynucleotide which encodes a protein of the invention in a Polymerase Chain Reaction.

In one embodiment, the method is capable of detecting the presence of *M. ptb*.

In yet another embodiment, the method is capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In another aspect, the invention provides a method of prophylactically or therapeutically treating an animal against Johne's disease.

In one embodiment, the method comprises administering to an animal a protein of the invention and/or a prophylactic or therapeutic composition comprising said protein of the invention, to engender in the animal a protective immunological response.

In another embodiment, the method comprises administering to an animal a protein of the invention and/or a therapeutic composition comprising said protein of the invention, to engender in the animal a protective response.

In a preferred embodiment, the prophylactic method comprises administering to said animal a vaccine composition comprising an acceptable diluent, carrier, excipient, or adjuvant, in addition to an immunologically protective amount of a protein or functionally equivalent variant or fragment thereof as described above.

In another aspect, the invention provides a kit comprising at least one of the following: a protein of the invention, an antibody or ligand that binds the protein of the invention, a oligonucleotide or polynucleotide primers capable of amplifying a polynucleotide which encodes a protein of the invention, for use in detecting the presence of *M. ptb*.

In one embodiment, the kit is capable of detecting the presence of *M. ptb* at a subclinical phase of Johne's disease.

In a still further aspect, the invention provides a cell-line, vector, or construct which includes a polynucleotide of the invention as defined above.

In a further aspect, the invention also provides a host cell incorporating a vector or construct of the invention capable of expressing a protein of the invention.

In one embodiment, the vector exists within the host cell as a plasmid.

In another embodiment, the vector is integrated into the genome of the host cell.

In a further aspect, the invention provides a method of transforming a cell with a polynucleotide which encodes a protein or functionally equivalent variant or fragment thereof as described above.

In a preferred embodiment, the host cell is capable of expressing a protein or analog thereof.

DESCRIPTION OF THE DRAWINGS

While the present invention is broadly as defined above, it also includes embodiments of which the following description provides examples. In particular, a better understanding of

the present invention will be gained through reference to the accompanying drawings in which:

Figure 1 depicts the amino acid sequence of the protein as inferred from the nucleotide sequence partially obtained from clone pTB-16, and subsequently amplified from the genome of *M. ptb* ATCC 53950 and sequenced

Figure 2 depicts the nucleotide sequence of the 705 base pair gene that encodes the protein.

Figure 3 shows the expression of the recombinant his-tagged protein from *M. smegmatis*. Protein was prepared from sonicated cells harbouring plasmid pMIP-p22 or pMIP12. Resulting soluble and insoluble fractions were electrophoresed in 15% SDS-PAGE gels. (a) Coomassie Blue stained gel. (b) Western blot analysis using 1:500 anti-histidine x 6 POD conjugated antibody. Lane M, molecular weight standard; lane 1, pMIP12 insoluble; lane 2, pMIP12 soluble; lane 3, pMIP-p22 insoluble; lane 4, pMIP-p22 soluble. Lanes 5 to 8 contain protein fractions collected from Ni^{+2}-affinity chromatography of the soluble fraction of cells harbouring pMIP-p22. Lane 5, unbound protein; lane 6, 40 mM imidazole elution; lane 7, 250 mM imidazole elution; lane 8, 1 M imidazole elution. Recombinant histidine-tagged protein is indicated by the arrows.

Figure 4 shows detection of antibody to the protein of the invention in sheep vaccinated with Neoparasec, such that of 11 sheep vaccinated with the live attenuated Neoparasec vaccine, 10 produced a strong antibody response to the protein. Western blots of recombinant protein were individually incubated with 1:1,000 dilution of sera. Anti-sheep IgG POD conjugated antibody was used at 1:20,000. (a) Mob 1 sheep. Lane M, molecular weight standard (kDa); lanes 1 to 6, pooled three and seven month post-vaccination sera from animals 124, 127, 129, 131, 132 and 136, respectively. Lanes 7 to 12, pooled one and two month pre-vaccination sera from the same animals. Lane 13, anti-histidine x 6 POD conjugated antibody control. (b) Mob 2 sheep. Lane M, molecular weight standard (kDa); lanes 1 to 5, post-vaccination sera from animals 507, 578, 587 (three month post-vaccination), 598* (two month post-vaccination), and 560* (one month post-vaccination),

respectively. Lanes 6 to 10, one month pre-vaccination sera from the same animals, respectively. Lanes 11 to 14, sera from unvaccinated animals 599, 569, 527 and 538, respectively, taken at the equivalent of three months post-vaccination.
* died after this time

Figure 5 shows the detection of antibody to the protein of the invention in individual sheep from a naturally infected flock. Western blots of recombinant protein were individually incubated with 1:500 dilution of serum. Anti-sheep IgG POD conjugated antibody was used at 1:40,000 dilution. Blots were developed by chemiluminescent detection. Lane M, molecular weight standard (kDa); lanes 1 to 14, sheep number 48, 44, 40, 36, 6, 13, 2, 23, 51, 32, 25, 27, 26 and 43, respectively. * animals diagnosed with Johne's disease.

Figure 6 shows the detection of antibody to the protein of the invention in naturally infected cattle. Western blots of recombinant protein were individually incubated with 1:500 dilution of serum. Anti-bovine IgG POD conjugated antibody was used at 1:20,000 dilution. Blots were developed by chemiluminescent detection. Lanes M, molecular weight standard (kDa); lane 1 anti-histidine x 6 POD conjugated antibody control; lanes 2 to 14, subclinical cattle which tested positive on at least one faecal culture (animal 24, 2, 275, 144, 327, 181, 115, 34, 49, 517, 168, 58, 68, respectively); lanes 15 and 16, clinically affected (symptomatic) cattle (animal 27 and 25); lanes 17 to 22, cattle that were negative on all faecal culture and serum ELISA tests (animal 211, 132, 193, 97, 174, 53). The position of the protein of the invention is indicated on both sides by arrows. See Table 1 for ELISA and faecal culture results.

Figure 7 shows IFN-γ induction using Ni^{+2}-affinity-enriched protein in Neoparasec-vaccinated sheep blood. Whole blood was incubated with 12.5 μg/ml Avian PPD in duplicate wells and 2.6 μg/ml, 0.64 μg/ml and 0.32 μg/ml Ni^{+2}-affinity enriched protein, in single wells. IFN-γ assays were performed as described. Results were expressed as "corrected" absorbance at 450 nm. For Avian PPD, this was defined as the average A450 nm of the Avian PPD-stimulated wells minus the average A450 nm of the PBS control wells for that animal. For each protein concentration, this was defined as the A450 nm of

the protein -stimulated well minus the average A450 nm of the PBS control wells for that animal. There was a significant difference ($p<0.01$) in the IFN-γ responses to the protein of the invention at all three concentrations between the Neoparasec vaccinated and unvaccinated group.

Figure 8 shows IFN-γ induction by purified recombinant protein in Neoparasec-vaccinated sheep blood. Whole blood was incubated in duplicate wells with 12.5 μg/ml Johnin PPD and 1 μg/ml size-exclusion purified protein of the invention. To demonstrate a concentration-dependent response, Neoparasec vaccinated animals 124, 127 and 129 and unvaccinated animals 128, 133 and 137 were similarly tested with 5 μg/ml purified protein. PBS was included as a negative control in duplicate wells. IFN-γ assays were performed as described. Results were expressed as "corrected" absorbance at 450nm, defined as the average A_{450nm} of the stimulated wells minus the average A_{450nm} of the PBS control wells for that animal.

Figure 9 shows detection of antibody to the protein of the invention from sheep vaccinated with *M. ptb* strain 316F culture filtrate. Western blots of recombinant protein were individually incubated with 1:500 dilution of serum as described. Anti-sheep IgG POD conjugated antibody was used at 1:40,000 dilution. Blots were developed by chemiluminescent detection. Lane M, molecular weight standard (kDa); lanes 1 to 5, one month post-vaccination animals 571, 513, 514, 512 and 551, respectively; lanes 6 to 10, pre-vaccination, same animals.

Figure 10 shows western blot detection of rabbit antibody raised to the protein of the invention. Ni^{+2}-affinity enriched recombinant protein was electrophoresed in SDS-PAGE gels and transferred to PVDF membranes for subsequent immunodetections with the following sera: lane 1, serum from rabbits immunized with the protein of the invention (1:1,000); lane 2, naïve rabbit serum (1:1,000); lane 3, control anti-histidine x 6 POD conjugated antibody (1:500). Lane M, molecular weight standard (kDa). Secondary anti-rabbit IgG POD conjugated antibody was used at 1:20,000. Blots were developed using chemiluminescent detection.

Figure 11 shows detection of native protein of the invention in Western blots of *M. ptb* strain 316F cell fractions and comparison to recombinant protein using rabbit antibody raised to the protein of the invention. (a) *M. ptb* cell fractions. Lane M, molecular weight standard (kDa); lane 2, 10 µl (0.5 mg) of 200 fold concentrated culture filtrate; lane 3, 10 µl of equivalent 200 fold concentrated soluble cell lysate fraction; lane 4, 10 µl of equivalent 200 fold concentrated insoluble cell fraction. (b) Native protein in *M. ptb* culture filtrate and recombinant protein from *M. smegmatis*. Lane M, molecular weight standard (kDa); lane 1, 5 µg culture filtrate; lane 2, 5 µg culture media only; lane 3, 1 µg Ni^{+2}-affinity purified recombinant protein from *M. smegmatis* lysate; lane 4, 0.1 µg Ni^{+2}-affinity purified recombinant protein from *M. smegmatis* lysate, incubated with 1:500 dilution anti-histidine x 6 POD conjugated antibody (control). Rabbit sera against the protein of the invention was used at 1:1,000 dilution. Secondary anti-rabbit IgG POD conjugated antibody was used at 1:20,000 dilution. Blots were developed using chemiluminescent detection as described. Arrows indicate the location of the protein of the invention.

Figure 12 shows PCR amplification of the gene encoding the protein of the invention from 13 isolates of *M. ptb*. PCR reactions were carried out using purified DNA as template and primer annealing at 62°C. Samples of 5 µl were electrophoresed in 1% agarose gels, stained with ethidium bromide and photographed under UV light. Lane M, DNA size standard; lanes 1 to 3, New Zealand farmed deer isolates type C1; lanes 4 to 6, New Zealand sheep isolates type S1; lane 7, New Zealand sheep isolate type S5; lanes 8 and 9, South African sheep isolates type I; lane 10, New Zealand cattle isolate type C1; lane 11, Faeroe Islands sheep isolate type S2. The DNA from the five IS*900*-typed strains were gifted by Dr. Desmond Collins, AgResearch, Wallaceville Animal Research Centre, New Zealand. Lane 12, ATCC 53950; lane 13, strain 316F; lane 14, negative control (water).

Figure 13 shows PCR amplification from 22 mycobacterial strains using primers designed to the ORF encoding the protein of the invention. PCR reactions were carried out using primers lpp-27fBam and lpp27-rKpn at two annealing temperatures. Samples of 5 µl were

electrophoresed on 1% agarose gels, stained with ethidium bromide and photographed under UV light. Simultaneous amplification of the 16S rRNA gene was carried out for each sample as a positive control (a single representative is shown). (a) 55°C annealing temperature. (b) 62°C annealing temperature. Lane M, DNA size standard; lane 1, *M. ptb* ATCC 53950; lane 2, *M. ptb* 316F; lane 3, *M. intracellularae*; lane 4, *M. scrofulaceum*; lane 5, *M. fortuitum*; lane 6, *M. terrae*; lane 7, *M. phlei*; lane 8, *M. smegmatis*; lane 9, *M. kansasii*; lane 10, *M. gordonae*; lane 11, *M. marinum*; lane 12, *M. bovis* 35746: lane 13, *M. bovis* 19210; lane 14, *M. bovis* KML; lane 15, *M. bovis* 35725; lane 16, *M. bovis* 35726; lane 17, *M. bovis* canine isolate; lane 18, BCG (Pasteur); lane 19, BCG (Glaxo); lane 20, BCG (Japan); lane 21, *M. tuberculosis* $H_{37}Ra$; lane 22, *M. tuberculosis* fur seal isolate; lane 23, negative control (water); lane 24, representative positive control 16S rRNA from *M. ptb* ATCC 53950. The 725 base pair PCR product is indicated by the white arrow. The red arrow shows a weak 725 base pair product produced in the *M. tuberculosis* complex at 55°C.

Figure 14 shows Southern blot analyses from genomic DNA of 13 mycobacterial strains using a probe to the gene encoding the protein of the invention. Approximately 1 μg of each DNA was digested with *Bam*HI, electrophoresed and transferred to nylon membranes as described. Probe DNA was labeled by incorporation of DIG-dUTP during PCR of the ORF encoding the protein of the invention. The blot was developed with CDP-star and exposed to film for 2 h. Lane 1, *M. bovis* BCG (Pasteur); lane 2, *M. tuberculosis* $H_{37}Ra$; lane 3, *M. bovis* (KML); lane 4, *M. intracellularae*; lane 5, *M. ptb* 316F; lane 6, *M. scrofulaceum*; lane 7, *M. gordonae*; lane 8, *M. kansasii*; lane 9, *M. phlei*; lane 10, *M. marinum*; lane 11, *M. terrae*; lane 12, *M. fortuitum*; lane 13, *M. ptb* ATCC 53950.

DESCRIPTION OF THE INVENTION

As broadly outlined above, in one aspect the invention provides a novel protein which has the amino acid sequence as set forth in Figure 1.

The molecular weight of the protein is 22kDa, as assessed by SDS-PAGE. Amino acid sequence analysis suggests the protein is a lipoprotein. The protein can be detected in the culture filtrate of *M. ptb*, suggesting it is a weakly associated envelope protein.

The protein of the invention can include its entire native amino acid sequence or can include only parts of that sequence where such parts constitute fragments which remain biologically active (active fragments). Such activity will normally be as an immunogen, but is not restricted to this activity.

Analogs of the protein and of its encoding polynucleotides are also within the scope of the present invention. Such analogs include functionally equivalent variants of the protein and of the polynucleotides described below.

The invention also includes within its scope functionally equivalent variants of the protein of Figure 1.

The phrase "functionally equivalent variants" recognises that it is possible to vary the amino acid sequence of a protein while retaining substantially equivalent functionality. For example, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent peptide is immunologically cross-reactive with and has at least substantially the same function as the original protein.

The functionally equivalent protein need not be the same size as the original. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment with additional amino acids. Active fragments may be obtained by deletion of one or more amino acid residues of full-length protein of the invention. It is also possible to substitute amino acids in a sequence with equivalent amino acids using conventional techniques. Groups of amino acids normally held to be equivalent are:

(a) Ala, Ser, Thr, Pro, Gly;

(b) Asn, Asp, Glu, Gln;

(c) His, Arg, Lys;

(d) Met, Leu, Ile, Val; and

(e) Phe, Tyr, Trp.

That equivalent may, for example, be a fragment of the protein containing from 8 to 50 amino acids, a substitution, addition or deletion mutant of the protein, or a fusion of the protein or a fragment or a mutant with other amino acids.

It is of course also possible (and expressly contemplated) that the bioactive peptides be or include any one of the octapeptides, nonapeptides, or decapeptides from the sequence.

Peptides which are, or include an octapeptide, nonapeptide or decapeptide from the protein of the invention from *M. ptb* ATCC 53950 are preferred.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989); Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Animal Cell Culture (R.I. Freshney, ed., 1987); Handbook of Experimental Immunology (D.M. Weir & C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller & M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); The Immunoassay Handbook (David Wild, ed., Stockton Press NY, 1994); Antibodies: A Laboratory Manual (Harlow et al., eds., 1987); and Methods of Immunological Analysis (R. Masseyeff, W.H. Albert, and N.A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993).

Polypeptide sequences may be aligned, and percentage of identical amino acids in a specified region may be determined against another sequence, using computer algorithms

that are publicly available. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. BLASTP software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The use of the BLAST family of algorithms, including BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F., *et al.* (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-34023.

Polypeptides of the invention also include homologous polypeptides having an amino acid sequence with at least 77% identity to the protein of the invention, preferably at least about 80% identity, more preferably at least about 90% identity, as well as those polypeptides having an amino acid sequence at least about 95% identical to the protein.

The invention also encompasses active fragments with bioactive functionality of the protein. Such active fragments may be obtained by deletion of one or more amino acid residues of full-length protein. Active fragments or portions of the protein may be ascertained by stepwise deletions of amino acid residues, from the N-terminal end or the C-terminal end or from within the polypeptide. If an amino acid is deleted and the bioactivity of the protein is not substantially reduced, then the amino acid may not comprise a portion of the active fragment. Further, polypeptides comprising an active fragment of the protein or its analog(s) are also encompassed in the invention.

A protein of the invention, its active fragments or other variants may be generated by synthetic or recombinant means (i.e. single or fusion polypeptides). Polypeptides, especially shorter polypeptides up to about 50 amino acids, are conveniently made by chemical synthesis using techniques well known to those of ordinary skill in the art. For example, to be prepared synthetically, the protein or its active fragments or other variants may be synthesised using any of the commercially available solid phase techniques such as the Merryfield solid phase synthesis method, where amino acids are sequentially added to a growing amino acid chain (see Merryfield, *J. Am. Soc.* 85:2146-2149 (1963)). Equipment for automative synthesis of peptides is also commercially available from

suppliers such as Perkin Elmer/Applied Biosystems, Inc and may be operated according to the manufacturers instructions.

The protein may also be produced recombinantly by inserting a polynucleotide (usually DNA) sequence that encodes the protein into an expression vector and expressing the peptide in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA molecule which encodes the recombinant peptides. Suitable host cells include prokaryotes, yeasts and higher eukaryotic cells. Preferably, the host cells employed are *Escherichia coli, Mycobacterium. smegmatis*, yeasts or a mammalian cell line such as COS or CHO, or an insect cell line, such as SF9, using a baculovirus expression vector. The DNA sequence expressed in this matter may encode the naturally occurring protein, fragments of the naturally occurring protein or variants thereof.

DNA sequences encoding the protein or fragments may be obtained by screening an appropriate *M. ptb* cDNA or genomic DNA library for DNA sequences that hybridise to degenerate oligonucleotides derived from partial amino acid sequences of the protein. Suitable degenerate oligonucleotides may be designed and synthesised by standard techniques and the screen may be performed as described, for example, in Maniatis *et al.* Molecular Cloning - A Laboratory Manual, Cold Spring Harbour Laboratories, Cold Spring Harbour, NY (1989). The polymerase chain reaction (PCR) may be employed to isolate a nucleic acid probe from genomic DNA, a cDNA or genomic DNA library. The library screen may then be performed using the isolated probe.

Variants of the protein may be prepared using standard mutagenesis techniques such as oligonucleotide-directed site specific mutagenesis.

Variants or homologues of the above polynucleotide sequences also form part of the present invention. Polynucleotide sequences may be aligned, and percentage of identical nucleotides in a specified region may be determined against another sequence, using

computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The BLASTN software is available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul, Stephen F, *et al* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402. The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu.pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in the W R Pearson and D.J. Lipman, "Improved Tools for Biological Sequence Analysis," *Proc. Natl. Acad. Sci. USA 85*:2444-2448 (1988) and W.R. Pearson, "Rapid and Sensitive Sequence Comparison with FASTP and FASTA," *Methods in Enzymology 183*:63-98 (1990).

Vectors and/or cells lines which express the protein, a functionally equivalent variant or active fragment thereof, have utility in their own right and also form part of the invention.

The invention also includes isolated nucleic acid molecules or polynucleotides that comprise a polynucleotide sequence encoding an ORF having at least about 80% identity, preferably at least about 85% identity, more preferably at least about 90% identity, as well as those polynucleotides having a nucleic acid sequence at least about 95%, 97%, 98%, or 99%, identical to the protein polynucleotide sequence set forth in Figure 2.

A raw nucleotide sequence 99% identical to that encoding the protein of the invention was located in the TIGR *Mycobacterium avium* subspecies *avium* database. This nucleotide

database was unannotated, the sequence had not been identified as an open reading frame, nor as a gene, and no protein-encoding utility had been ascribed to the raw nucleotide sequence. Accordingly, the applicants are the first to teach the identification of this nucleotide sequence as a protein-encoding gene.

All sequences identified as above qualify as "variants" as that term is used herein.

Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C. Such hybridizable sequences include those which code for the equivalent protein from sources (such as other Mycobacterium) other than *M. ptb*.

While the above synthetic or recombinant approaches can be taken to produce the protein of the invention, it is however practicable to obtain the protein by isolation from *M. avium* and/or a subspecies or strain thereof, or a culture filtrate from *M. avium*, and/or a subspecies or strain thereof.

Particularly suitable subspecies of *M. avium* from which the protein can be isolated are *M. ptb*, with *M. ptb* strain ATCC 53950 being presently preferred.

Once obtained, the protein is readily purified if desired. This may involve affinity chromatography in which the immunogenic nature of the protein is important. Other approaches to purification (eg. gel-filtration or anion exchange chromatography) can however also be followed. Where the protein or fragment is produced in the form of a fusion protein, the carrier portion of the fusion protein can prove useful in this regard.

Furthermore, if viewed as desirable, additional purification steps can be employed using approaches which are standard in this art. These approaches are fully able to deliver a

highly pure preparation of the protein. Preferably, the protein preparation comprises at least about 50% by weight of the protein, preferably at least about 80%, preferably at least about 90%, and more preferably at least about 95% by weight of the protein.

The purification procedure will of course depend on the degree of purity required for the use to which the protein or fragment is to be put.

Once obtained, the protein and/or its active fragments and/or its functionally equivalent variants can be formulated into a composition. The composition can be, for example, a therapeutic composition for application as a veterinary pharmaceutical, a vaccine, or a diagnostic composition. For these purposes it is generally preferred that the protein be present in a pure or substantially pure form. Again, standard approaches can be taken in formulating such compositions (see for example, Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing (1990)).

In one embodiment, the protein, its functionally equivalent variants or active fragments are employed as antigens to elicit a host-protective response in an animal. It will be appreciated that in accordance with this embodiment, the antigen of the invention can be administered either alone or in the form of a composition including a vaccine preparation comprising the antigen as the active ingredient together with a pharmaceutically acceptable diluent, carrier, excipient, or adjuvant.

Examples of suitable adjuvants known to those skilled in the art are saponins (or derivative or related material), muramyl dipeptide, trehalose dimycollate, Freund's complete adjuvant, Freund's incomplete adjuvant, other water-in-oil emulsions, double emulsions, dextran, diethylaminoethyl-dextran, potassium alum, aluminium phosphate, aluminium hydroxide, bentonite, zymosan, polyelectrolytes, retinal, calcium phosphate, protamine, sarcosine, glycerol, sorbitol, propylene glycol, fixed oils and synthetic esters of higher fatty acids. Saponins are generally preferred.

The antigen of the invention may also be treated in any conventional way to enhance its stability or to conserve or potentiate its immunogenic efficacy. For example, the antigen may be treated with a suitable inhibitor, modifier, crosslinker, or denaturant in such a way as to enhance its immunogenicity.

In addition, the antigen can be administered in combination with other therapeutic agents.

It is also possible to include an additional immunogen in the solution or composition for administration as an vaccine. Such an immunogen will generally be a Th1 type immune response inducing substance.

An immunogenic composition including a vaccine of the invention can be administered to the animal by any of those methods known in the art. However, the preferred mode of administration is parenteral. The term "parenteral" is used herein to mean intravenous, intramuscular, intradermal, and subcutaneous injection. Most conveniently, the administration is by subcutaneous injection.

Despite the preference for parenteral administration, it is by no means intended to exclude administration of the immunogenic composition in other forms.

The amount of composition administered to the animal to be treated will depend on the type, size and body weight of the animal as well as on the immunogenicity of the immunogenic composition. Conveniently, in the case of a vaccine, the vaccine is formulated such that relatively small dosages of vaccine (1 to 5 mL) are sufficient to protect the animal to which they are administered. The term "animal" as used herein includes ruminants such as cattle, sheep, deer, buffalo, camelids, antelope, and goats.

In addition to the vaccine embodiments described above, it will be understood that a live vaccine could also be employed to protect a host against *M. ptb* infection or Johne's disease. In such a live vaccine, the DNA molecule of the invention encoding the protein, functionally equivalent variant or active fragment thereof, is incorporated into the genome

of an attenuated carrier organism. A number of carrier organisms are known in the art which are suitable for this purpose with examples being Salmonella and Vaccinia virus.

The live vaccine of this embodiment can be formulated in accordance with methods known in the art. Similarly, the live vaccine can be administered parenterally, or by conventional methods to have its effect via the gastrointestinal tract.

It is well known by those skilled in the art that *M. ptb* is the causative pathogen of Johne's disease. A further use of the protein of the invention, its functionally equivalent variant or active fragment thereof, is in the field of diagnosis of *M. ptb* infection and/or Johne's disease. More particularly, it will be understood by those persons skilled in the art that the protein, functionally equivalent variant or active fragment thereof, may be employed as highly specific diagnostic reagents for detection of the presence of *M. ptb* in an animal suspected of harbouring the disease. The diagnostic procedure employed to detect *M. ptb* and/or Johne's disease may involve the detection of delayed type hypersensitivity to the protein of the invention by skin test or the like, or may involve an assay of body fluid obtained from the animal.

Similarly, the diagnostic procedure employed to detect *M. ptb* and/or Johne's disease may involve the use of the protein of the invention as a reagent in an IFN-γ EIA test.

It will be understood by those persons skilled in the art that the detection system selected may employ the protein, functionally equivalent variant or active fragment thereof, alone as diagnostic reagents.

However, in other systems such diagnostic reagents will include suitable carrier or marker substances (either chemical, enzymic or radiochemical) in the detection of antibodies by agglutination, radioimmunoassay, fluorescence or enzyme immunoassay techniques. A preferred antibody detection technique is ELISA.

Another use for the polypeptides of the invention is the generation of antibodies, including monoclonal antibodies. Polypeptides of the invention are used as immunogens to immunize mice. Splenocytes (including lymphocytes) are obtained from the immunized mice. Hybridomas are prepared from the lymphocytes and immortalized myeloma cells using the general somatic cell hybridization technique of Kohler, B. and Milstein, C. Nature 256:495-497 (1975). Other modified methods, for example by Buck, D. W., et al., In Vitro, 18:377-381 (1982) may also be used. Available myeloma lines, including but not limited to X63-Ag8.653 and those from the Salk Institute, Cell Distribution Center, San Diego, Calif., USA, may be used in the hybridization. The technique involves fusing the myeloma cells and lymphoid cells using a fusogen such as polyethylene glycol, or by electrical means well known to those skilled in the art. After the fusion, the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. Any of the media described herein, supplemented with or without serum, can be used for culturing hybridomas that secrete monoclonal antibodies. As another alternative to the cell fusion technique, EBV immortalized B cells are used to produce the monoclonal antibodies of the subject invention. The hybridomas are expanded and subcloned, if desired, and supernatants are assayed for anti-immunogen activity by conventional immunoassay procedures (e.g., radioimmunoassay, enzyme immunoassay, or fluorescence immunoassay).

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal antibodies may be isolated from the culture media or body fluids, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired. Undesired activity if present, can be removed, for example, by running the preparation over adsorbants made of the immunogen attached to a solid phase and eluting or releasing the desired antibodies off the immunogen.

Polypeptides of the invention may also be used as immunogens to immunize other animals (i.e., rats and rabbits) to generate polyclonal antibodies. Methods of producing polyclonal antibodies and isolation and purification thereof is known in the art. See, for example,

Harlow and Lane (1987). Other suitable techniques for preparing antibodies involve *in vitro* exposure of lymphocytes to the antigen or alternatively to selection of libraries of antibodies in phage or similar vectors. See, for example Huse *et al.*, 1989.

Also, recombinant antibodies may be produced using procedures known in the art. See, for example, US Patent 4,816.567.

The antibodies may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently a substance which provides a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in the literature.

Antibodies as above to the protein of the invention can therefore be used to detect or monitor the presence of the protein in an animal or in protein quantification assays. Further, antibodies to the protein of the invention can be used to measure levels of the protein in an animal, either at one fixed time point or over a period of time to monitor fluctations in protein levels. Such antibodies can also be used to measure levels of the protein in an animal to which drugs, vaccines, or other therapeutic or prophylactic bioactives have been administered. In such assays, any convenient immunological format can be employed. Such formats include immunohistochemical assays, RIA, IRMA and ELISA assays.

The assays can be conducted in relation to any biological fluid which does, or should, contain the protein of the invention. Such fluids include blood, serum, plasma, urine and cerebrospinal fluid.

Antibodies as above to the protein of the invention can therefore be used to detect or monitor the presence of *M. ptb* and/or Johne's disease in an animal or in *M. ptb* quantification assays. Further, such antibodies can be used to measure levels of *M. ptb* in an animal, either at one fixed time point or over a period of time to monitor fluctations in *M. ptb* levels. Such antibodies can also be used to measure levels of *M. ptb* in an animal to

which drugs, vaccines, or other therapeutic or prophylactic bioactives have been administered. In such assays, any convenient immunological format can be employed. Such formats include immunohistochemical assays, RIA, IRMA and ELISA assays.

The assays can be conducted in relation to any biological fluid which does, or should, contain the protein of the invention, a functionally equivalent variant or active fragment thereof, and/or *M. ptb*. Such fluids include blood, serum, plasma, urine and cerebrospinal fluid.

Antibodies, monoclonal or polyclonal, against the protein of the invention may be used for diagnosis or for therapeutic purposes. Antibodies may be used by themselves or attached to a solid substrate, such a column or a plate. Antibodies which are attached to a solid substrate may be used for assays, for example ELISA, or as a standard in other assays. Antibodies against the protein of the invention are also useful for isolation, purification, and quantitation of the protein of the invention.

Those skilled in the art will appreciate that the functional equivalents of antibodies, such as antibody fragments and/or f_{ab} molecules are also considered herein.

The antibodies can also be included in assay kits. Such kits can contain, in addition, a number of optional but conventional components, the selection of which will be routine to the art skilled worker. Such additional components will however generally include a protein reference standard, which may be the protein of the invention itself or an analog (such as a fragment).

It will also be appreciated that antibodies such as described above can, in some circumstances also function as antagonists of the protein of the invention by binding to the protein and partly or completely interfering with its activity.

Similarly, those skilled in the art will appreciate that other ligands that bind the protein of the invention, a functionally equivalent variant or active fragment thereof, may be used in a fashion analogous to the antibodies to the protein described above.

Aspects of the invention will now be described with reference to the following non-limiting examples.

EXAMPLE 1

To identify potential exported proteins of *M. ptb*, a library of alkaline phosphatase (*phoA*) gene fusions was constructed in the vector pJEM11 and expressed in *Escherichia coli* and *Mycobacterium smegmatis*.

Materials and Methods

Extraction of DNA from M. ptb

Approximately 3mg of lyophilised *M. ptb* (New Zealand field isolate ATCC 53950) was resuspended in 0.6ml of extraction buffer (100mM NaCl, 25mM EDTA (pH 8.0), 10mM Tris.Cl pH 8.0, 0.5%(w/v) SDS) before adding 200µg of proteinase K (Roche Molecular Biochemicals, Germany). The mixture was incubated at 50°C for 18h and then 100µl of 5M NaCl and 120µl of 6.7%(w/v) cetyltrimethylammonium bromide (Aldrich Chemical Company, USA) in 0.5%(w/v) NaCl was added. The digest was mixed with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol and centrifuged at 15,800 x g for 5min. The aqueous phase was collected and the phenol:chloroform:isoamyl alcohol extraction was repeated, as above. The aqueous phase was mixed with an equal volume of chloroform and centrifuged again. The DNA was precipitated with the addition of 1 volume of 100% isopropanol to the collected aqueous phase. After 18h at -20°C, the DNA was pelleted by centrifugation at 15,800 x g for 30min at 4°C. The pellet was washed with 1ml of 70% ethanol, air dried at room temperature and resuspended in 100µl of TE buffer containing 100µg/ml RNAse A (Life Technologies Inc., USA) and incubated for 18h at 37°C. The DNA concentration was calculated based on absorbance at 260nm. To check

the condition of the DNA, an 8µl sample (~11µg) was electrophoresed on a 0.7% agarose gel, stained with ethidium bromide and visualised under UV light.

Preparation of pJEM11 vector DNA

pJEM11 plasmid DNA was purified from transformed *E. coli* DH10B cells. The plasmid DNA was quantitated based on its absorbance at 260nm. Eleven micrograms of plasmid was digested to completion with 2.5units of *Bam*HI at 37°C for 2h and was purified by agarose gel extraction. The resulting digested plasmid DNA was dephosphorylated using 2 units of alkaline phosphatase (Boehringer Mannheim, Germany) for 1h at 37°C to prevent recircularisation of the plasmid. The DNA was then purified by agarose gel extraction.

Partial digestion of M. ptb *genomic DNA*

A partial digest of *M. ptb* genomic DNA was performed on 2.8µg of the DNA using 0.05 units *Sau*3A in a volume of 25µl at 22°C. From this, 6 x 4µl samples were removed at 30s intervals and were electrophoresed on a 1% agarose gel. DNA ranging in size from approximately 200 - 3,000 base pairs was extracted from the 1.5min and 2.0min digestion lanes from the agarose gel. The resulting DNA fragments were eluted in a final volume of 50µl of TE buffer. A 10µl sample was run on a 1% agarose gel to check recovery.

Ligation of size selected M. ptb *genomic DNA and pJEM11 and transformation into* E. coli

Approximately 1µg of *Bam*HI, alkaline phosphatase-treated pJEM11 vector DNA and 0.7µg of *Sau* 3A partially digested *M. ptb* DNA were ligated using 1.0unit of T4 DNA ligase in a total volume of 35µl at room temperature for 30min. The mixture was dialysed and electroporated into 100µl of *E. coli* DH10B cells. To this, 500µl of LB broth was added and incubated for 1h at 37°C. Aliquots of approximately 70µl each were plated onto nine LB/kan/BCIP plates and incubated at 37°C for approximately 18h. A representative plate was selected and colonies were counted on a quarter of the plate to estimate the total number of resulting transformants. The total number of blue transformants was also counted. Blue *E. coli* colonies were restreaked onto LB/kan/BCIP plates for confirmation of $PhoA^+$ phenotype. Each of these colonies was cultured in LB/kan broth for glycerol storage at -70°C.

Plasmid isolation from the E. coli *recombinant library*

The recombinant plasmid library was then isolated from *E. coli*. To each of the nine plates, 1.6ml of LB/kan broth was added and the colonies were resuspended with the aid of a rubber spatula. The mixtures from each plate were transferred to 2ml microtubes and the plasmid DNA was extracted using a BRESAspin Plasmid Mini Kit (Bresatec, Australia) with the modification that all reagent volumes were doubled due to the high concentration of cells. Each of the nine plasmid preparations was eluted into 50µl of TE buffer, for a total volume of 450µl. The plasmids were stored at -20°C until used.

Transformation of the recombinant plasmids into M. smegmatis

The recombinant plasmids were then transformed into *Mycobacterium smegmatis* mc^2155. Two 10µl aliquots of the plasmid mixture were dialysed and used to transform two 100µl aliquots of electrocompetent *M. smegmatis* cells. To each of the resulting transformations, 500µl of LB broth was added and the cells were incubated at 37°C for 2h. From this, 80µl samples were spread onto 15 LB/kan/BCIP plates, which were then incubated at 37°C. After five days, the plates were transferred to 4°C for a further 54 days. To estimate the total number of colonies (blue and white), a quadrant of a representative plate was counted. Over the 59 days, blue colonies were picked daily and designated numerically, as they appeared. These were restreaked onto fresh LB/kan/BCIP plates and grown at 37°C until they turned blue and then were transferred to glycerol for storage at -70°C.

Sequencing of DNA inserts encoding putative exported proteins

To obtain plasmid DNA for sequencing of inserts, the pJEM11 constructs were first transferred from individual *M. smegmatis* colonies to *E. coli* DH10B cells. Prior to sequencing, the clones were first screened for presence of unique inserts by digestion of the plasmids with restriction endonucleases *Kpn* I and *Apa* I, which flank the DNA inserts in pJEM11. Unique inserts were then selected for sequencing.

Results

Construction of an M. ptb pJEM11 expression library

In order to identify *M. ptb* gene sequences encoding exported proteins, a library of *M. ptb phoA* fusions was created in the vector pJEM11 as described above and expressed in *E. coli* and *M. smegmatis*.

Since *M. smegmatis* mc^2155 has a 10^4 lower rate of transformation efficiency as compared to *E. coli* (Snapper *et al.*, 1990), the plasmid library was first transformed into *E. coli* to ensure the highest possible proportion of constructs could be recovered.

After 18 h at 37°C a representative *E. coli* plate had 1,200 colonies. This resulted in an estimated total of 10,800 colonies from nine plates. Of these, 17 colonies were blue. These were designated Eco-1 to Eco-17. Upon a further 18 h storage of the library at 4°C, seven more blue *E. coli* colonies resulted. These were designated Eco-18 to Eco-24. Thus, a total of 24 blue *E. coli* colonies, representing 0.2% of the library were obtained.

Expression of the library in M. smegmatis

A sample of the plasmid collection was used to transform *M. smegmatis*. After three days incubation at 37°C on LB/kan/BCIP plates, the first blue *M. smegmatis* colony appeared and was designated pTB-1. Over the next two days, a further 45 blue colonies appeared, designated pTB-2 to pTB-46. The plates were transferred to 4°C and 473 more blue colonies appeared over 54 days. A total of 519 blue *M. smegmatis* clones were collected. The total number of *M. smegmatis* colonies was estimated to be 60,000, of which 0.9% were blue.

A six-fold increase in the number of colonies was obtained in *M. smegmatis* (60,000) compared to *E. coli* (10,800). This was a result of amplification of the plasmids in *E. coli* prior to transfer to *M. smegmatis*. Multiple copies of the plasmids were therefore expected to be present in the *M. smegmatis* library. This expectation was confirmed by analysis of the pJEM11 plasmid constructs. The average insert size was calculated from $PhoA^+$

Materials and Methods

PCR amplification of the gene from M. ptb

Genomic DNA extracted from *M. ptb* ATCC 53950, prepared as described above, was used as a template for PCR amplification of the gene encoding the protein of the invention. Oligonucleotide primers were designed to the 5' and 3' ends of the entire ORF and are shown below. The forward primer lpp27-fBam, was designed to the 5' end of the predicted ORF, and the reverse primer lpp27-rKpn, was designed to the 3' end of the ORF. The TGA stop codon was omitted to allow read-through to produce the histidine x 6 tag coded by the vector.

lpp27-fBam	5' GATGGGATCCATGCAGACCCGCCGCCGCCT
lpp27-rKpn	5' TGAGGGTACCCGAGCTCACCGGGGGCTTGG

PCR was done using 1µl of a 1:10 dilution of genomic DNA template in a volume of 50µl. The conditions used were an initial melting temperature of 95°C for 10min followed by 35 cycles of 94°C for 30s, 55°C for 30s, 68°C for 1min, and a final extension at 68°C for 10min using platinum *Pfx* polymerase, in the presence of 10%(v/v) dimethlysulfoxide.

Cloning of the open reading frame

For expression of the gene encoding the protein of the invention in *M. smegmatis*, the ORF was cloned into the vector pMIP12. The 725 base pair PCR product was extracted from a 1% TAE agarose gel. The purified product was eluted in 30µl of distilled water and a 6µl sample was run on a 1% agarose gel to check its recovery. The remaining DNA was digested with 40units of *Kpn*I in 40µl at 37°C overnight. This was followed by digestion with *Bam*HI in a total volume of 50µl at 37°C overnight. The digested product was purified and resuspended in 30µl of sterile water using a QIAgen gel extraction kit. From this, 6µl was electrophoresed on a 1% TAE agarose gel to estimate its concentration. The remainder was used for ligation with pMIP12, as described below.

The vector pMIP12 was isolated from previously transformed *E. coli* DH10B cells. Plasmid DNA from two 4ml cultures grown in LB/kan broth was resuspended in 100µl of distilled water. DNA concentration was quantitated by absorbance at 260nm. Approximately 2.6µg of plasmid DNA was digested with 20units of *Kpn*I in 20µl for 4h at 37°C. To confirm completion of digestion, 1µl was removed and electrophoresed on a 1% TAE agarose gel and photographed. To the remaining 19µl, 30units of *Bam*HI was added and the volume was brought up to 30µl with water. The mixture was incubated for 18h at 37°C. The digested vector was electrophoresed on a 1% low melting point agarose gel (SeaPlaque GTG agarose, FMC BioProducts, Rockland, ME, USA) in TAE buffer and ethidium bromide stained for visualisation. The gel slices were cut out in a minimal amount of agarose, transferred to a 1.5ml microfuge tube and melted at 68°C for 10min. From this mixture, 6µl was removed and immediately added to a microfuge tube containing 24µl of insert DNA, prepared as described above. To this was added 1unit of T4 DNA ligase in a total volume of 40µl and the mixture was incubated at 14°C overnight. This was dialysed and electroporated into 35µl of *E. coli* ElecrtoMax DH10B cells. To this, 200µl of LB broth was added and the mixture was incubated at 37°C for 1h. Of this, 50µl was spread onto a LB/kan plate and incubated overnight at 37°C.

To confirm the presence of the insert, PCR was carried out on five of the resulting colonies using the pMIP12 primers BlaF3 and R2 (see below), in the presence of *Taq* polymerase in 20 µl. Eight microlitres from each PCR was electrophoresed on a 1% TAE agarose gel and ethidium bromide stained. The forward primer BlaF3 binds at approximately 150 base pairs within the *blaF** promoter and is designed to the coding strand. The reverse primer R2 binds 44 base pairs from the *Kpn* I site and is designed to the complementary strand of the transcriptional terminator. The expected size of the PCR product for the insert was approximately 900 base pairs.

BlaF3	5' TCGCGGGACTACGGTGCC
R2	5' TCGAACTCGCCCGATCCC

constructs analysed by agarose gel electrophoresis. The plasmids were digested with *Apa* I and *Kpn* I, whose recognition sites flank the *Bam* HI site in the vector used for cloning. Only unique inserts were included in calculations. From the 128 plasmids analysed, the average insert size was 1,700 base pairs. The inserts generally ranged in size from 300 - 3,000 base pairs with a median of 1,400 base pairs.

Insert DNA was fully or partially sequenced from 63 individual clones. Of these, 33 were unique in sequence and the rest were redundant. The partial gene sequences adjacent to *phoA* were used to search DNA and protein databases. Twenty-five of the 33 sequences obtained had similarities to other mycobacteria or *Streptomyces* genes and included identities with a copper/zinc superoxide dismutase, a cutinase, a penicillin-binding protein, a serine/threonine protein kinase and three lipoproteins. One sequence was identical to the previously characterised gene encoding the *M. ptb* 34 kDa protein. Seven sequences had no significant similarities to any of the sequences on the databases.

N-terminal regions were determined for 21 of the 33 translated open reading frames fused to *phoA*. These sequences were further analysed for the presence of conserved signal peptide elements and transmembrane-spanning regions. Twelve had evidence of signal sequences and four had predicted transmembrane segments. The remaining five had no evidence of characteristic hydrophobic stretches of amino acids that might function as transmembrane segments. Of the twelve sequences with indefinable N-termini, eight had predicted transmembrane-spanning regions.

A putative lipoprotein identified in this study was selected for further investigations.

EXAMPLE 2

An exported 23 kDa protein, identified as described above, was characterised and assessed for its immunoreactivity. The open reading frame encoding the protein was cloned and the protein was expressed in *M. smegmatis* as a C-terminal polyhistidine-tagged recombinant protein as described below.

From a resulting PCR-positive colony, plasmid DNA was extracted from 4 ml of broth culture and resuspended in 50 µl of 10 mM Tris (pH 8.0). Eight microlitres of plasmid was digested with 10 units of *Kpn* I in 10 µl at 37°C overnight. This was followed by digestion with 10 units of *Bam* HI in a final volume of 25 µl at 37°C overnight. One microlitre was electrophoresed on a 1% TAE gel and photographed after ethidium bromide staining. To confirm the insert identity and the correct insertion for expression, plasmid was used as template for sequencing using the primers Bla3 and R2.

Expression and purification of recombinant protein from M. smegmatis

For transfer of pMIP-p22 into *M. smegmatis*, 1 µl of the plasmid DNA was electroporated into 20 µl of *M. smegmatis* cells. A 250 µl aliquot of cells was spread on a LB/kan plate and incubated at 37°C for three days. To confirm the presence of pMIP-p22, one colony was picked for PCR analysis using the primers R2 and Bla3 as described above, in a volume of 100 µl. The resulting 900 base pair product was gel-extracted and sequenced using the same primers.

For preparation of recombinant protein, a single colony of pMIP-p22-transformed *M. smegmatis* was picked and inoculated into 15 ml of Sauton's broth or modified Middlebrook 7H9 broth containing kan and grown at 37°C with vigorous shaking for approximately three days. This was used to inoculate 600 ml of the same media, and the culture was grown and harvested and lysates were prepared as described.

The resulting sonicate supernatant was used in Ni^{+2}-affinity chromatography, employing four 5 ml columns connected in series and attached to a peristaltic pump. Imidazole concentrations of 40 mM, 250 mM and 1M were used, in the first instance, to determine the elution profile of the recombinant protein. Samples from each collected elution were used in Western blot analyses to determine where the majority of the recombinant protein eluted. Having determined this, the procedure was repeated using two washes of 100 mM of imidazole prior to the 250 mM elution, to further purify the recombinant protein. Pooled fractions were concentrated as described, in preparation for Western blotting, IFN-γ assays or size exclusion chromatography.

N-terminal protein sequencing

Automated Edman degradative N-terminal sequencing was carried out by Massey University Protein Sequencing Services using a pulse liquid phase sequenator (Model 476A, Applied Biosystems, USA). This instrument performs fully automated sequencing by sequential removal of the N-terminal amino acid as a phenylthiohydantoin-derivative. Derivatives were separated by HPLC and the data collected and analysed using a Model 610A Data Analysis Module (Applied Biosystems, USA). Protein to be sequenced was electrophoresed on 15% SDS-PAGE gels and transferred to PVDF membrane as described. Following transfer, the membrane was stained with Ponceau S and the desired band(s) were excised and destained in distilled water. Approximately 1 pmol (25 μg for a protein of 25 kDa) of protein was used for sequencing.

Results

Expression and purification of recombinant protein from M. smegmatis

In order to increase the likelihood of producing the protein of the invention in a form resembling that from the original host (*M. ptb*), it was expressed in the fast-growing species *M. smegmatis*. The ORF encoding the protein of the invention was cloned into the vector pMIP12 and used to transform *M. smegmatis*. To aid purification and detection of the recombinant protein, the gene was expressed with a C-terminal histidine x 6 tag from the vector pMIP12. As shown in Figure 3, the recombinant protein was detected from cell sonicates in both the soluble and insoluble fractions by Western blot analysis using a monoclonal anti-histidine x 6 POD conjugated antibody. The recombinant protein was clearly recognised by the antibody (Figure 3b) with no other signal obtained, confirming specificity for the histidine x 6 tag. The protein was further isolated from the soluble fraction by Ni^{+2}-affinity chromatography followed by elution with imidazole as described above. Recombinant protein could be seen in the 250 mM imidazole elution in SDS-PAGE gels with Coomassie Blue staining (see Figure 3a). Western blot analysis showed the 250 mM imidazole elution contained most of the protein, with a slightly smaller amount present in the 40 mM elution and none was detected in the 1 M elution. There was

a small amount of the protein still present in the flow-through, indicating that not all of the protein was bound to the column. This may be because the column was saturated with bound protein.

The apparent molecular weight of the recombinant protein from SDS-PAGE was 23 kDa, which was smaller than the calculated weight of 25.9 kDa for the full-length recombinant protein, expressed from pMIP12. This suggested that the full-length protein may have been cleaved at the N-terminus, as was predicted from its amino acid sequence, and so N-terminal sequencing of the 23 kDa band was carried out, as described above. The resulting amino acid sequence obtained, **LIAGCS**, was consistent with an N-terminal cleavage predicted at amino acid position 19/20 in the native protein by SignalP (see Figure 1, amino acids 20 – 25). The theoretical size of the mature recombinant product based on this cleavage was 23.6 kDa and was similar to the apparent molecular mass from SDS-PAGE.

EXAMPLE 3

Materials and Methods

Western blot analyses of antibodies in animal sera

Western blots were carried out according to methods well known to those skilled in the art. For detection of antibody to the protein of the invention from animal sera, identical amounts of recombinant protein (approximately 0.5 μg) were electrophoresed in individual lanes, alongside a molecular weight standard, on SDS-PAGE gels. The protein was transferred to PVDF or nitrocellulose membranes and stained with Ponceau S to visualise the bands. The lanes were numbered at the top of the membrane and then cut into individual strips.

For immunodetection, single strips were incubated with serum in individual small glass screw-capped bottles in 2 ml of blocking buffer. The strips remained in the bottles for washing and were then were pooled in plastic trays for incubation with appropriate secondary antibody and washing. To avoid cross-contamination, strips from different

experimental groups, eg. unvaccinated and vaccinated, were pooled separately during this stage. The following secondary POD conjugated anti-IgG heavy and light chain antibodies were used: goat anti-rabbit (A 6154), donkey anti-sheep (A 3415), rabbit anti-bovine (A 7414), (Sigma, USA).

Results

Humoral immune responses to the protein

To investigate immune responses to the protein of the invention, Neoparasec-vaccinated animals were used in the first instance. It was hypothesised that vaccinated animals would make an immune response to antigens possessed by the bacteria in the vaccine. In this respect, they may be similar to infected animals and therefore could be used to evaluate the immunogenicity of the protein of the invention. *M. ptb* vaccinated cattle and sheep have similarly been used to evaluate various mycobacterial proteins (Valentin-Weigand & Moriarty, 1992; Koets *et al.*, 1999; 2001).

Serum samples from Neoparasec-vaccinated and naturally infected sheep were used in individual immunoblot assays to determine their humoral response to the protein of the invention. A high level of antibody to the protein of the invention was produced by 10 of the 11 Neoparasec-vaccinated sheep in Western blots analyses, demonstrating the protein was immunogenic, as shown in Figure 4. There was a very faint band produced by sera from three of the sheep (507, 578 and 560) prior to vaccination and also in two naïve animals (599 and 569). Control sera from the remaining animals prior to vaccination and from unvaccinated control animals did not react with the protein (not all are shown).

To investigate if antibody to the protein of the invention was present in naturally infected animals, Western blots were probed with sera from sheep and cattle belonging to flocks and herds known to have Johne's disease present. Results with sheep sera are shown in Figure 5. A total of five of 14 sheep had antibody to the protein of the invention. Antibody to the protein of the invention was detected in two of the five sheep with confirmed Johne's disease and in two sheep that showed no evidence of Johne's disease.

A weak band was produced with one animal (26) that had a single suspicious lesion without acid-fast organisms in an examined lymph node but was not confirmed as having Johne's disease. Immunoblots were repeated several times with consistent results.

Figure 6 shows the results of Western blot analysis using cattle sera. A summary of the results for serum ELISA, faecal culture and Western blot analysis is shown in Table 1. Antibody to the protein of the invention was present in sera from 11 of 13 subclinically infected cattle that were positive on at least one previous faecal culture. A variety of strong and weak bands were produced. Three of the four cows that were positive on all ELISA and faecal culture tests (24, 144 and 49 but not 2) also produced major bands with the protein of the invention. Two subclinically infected cows (181 and 68) did not have detectable antibody to the protein of the invention and were also negative on the last ELISA and previous faecal culture. However, other animals with similar test results, such as 58 and 34, produced strong bands with the protein of the invention. Antibody to the protein was present in four subclinically infected cows (327, 517, 58, 115) that were negative on all three serum ELISA tests. Antibody to the protein was also present in both clinically affected cows (27 ands 25). Cow 27 produced a very strong band with the protein of the invention. Of the six cows that were negative on all ELISA tests and faecal cultures, one (53) produced a weak band with the protein of the invention.

EXAMPLE 4

Materials and Methods

IFN-γ assays

IFN-γ assays were performed using the whole blood Bovigam™ EIA bovine interferon test, which is suitable for the detection of ovine IFN-γ. The test was performed as per the manufacturer's (Commonwealth Serum Laboratories, Australia) instructions, with some modifications. Briefly, blood samples were collected in lithium heparin tubes and processed within 4 hours of collection.

Table 1. Summary of results for detection of *M. ptb* by serum ELISA, faecal culture and Western blot analysis

Animal #	ELISA 1	ELISA 2	ELISA 3	Faecal culture 1	Faecal culture 2	protein
24	+	+	+	+	+	+
2	ND	+	+	+	+	+
275	+	-	+	+	+	+
144	+	+	+	+	+	+
327	-	-	-	+	+	+
181	+	+	-	+	-	-
115	-	-	-	-	+	+
34	-	+	-	+	-	+
49	+	+	+	+	+	+
517	-	-	-	+	+	+
168	ND	-	+	+	+	+
58	-	-	-	+	-	+
68	-	-	-	+	-	-
27#	ND	ND	ND	ND	ND	+
25*	ND	ND	ND	ND	ND	+
211	-	-	-	-	-	-
132	-	-	-	-	-	-
193	-	-	-	-	-	-
97	-	-	-	-	-	-
174	-	-	-	-	-	-
53	-	-	-	-	-	+

Serum ELISA and faecal culture were carried out and interpreted as positive or negative by AgResearch, Wallaceville, Lower Hutt, New Zealand. Sera and faecal culture tests were done in six-monthly intervals. Sera used for Western blot analysis was from the last collection date, corresponding to ELISA 3.
ND = not done
* Johne's disease diagnosed on clinical signs and gross pathology
# Johne's disease diagnosed on clinical signs and acid-fast organisms in faeces

For each antigen to be tested, 1ml aliquots of blood were dispensed into 24-well tissue culture trays. Routinely, antigens were tested in duplicate. Each antigen was added in a standard volume of 67 µl to the blood aliquots and mixed for 5 min on a rotating platform

shaker. The trays were then incubated 22 h at 37°C in a humidified atmosphere with 5% CO_2. From each blood/antigen aliquot, 200 µl of plasma was harvested and stored at -20°C in 96-well plates for subsequent testing. The plasma samples were assayed singly for IFN-γ using Bovigam™ EIA plates according to the manufacturer's instructions. Absorbance readings were carried out on a MAXline, Vmax® (Molecular Devices Corp., USA) kinetic microplate reader at 450 nm.

Johnin PPD or Avian PPD (Commonwealth Serum Laboratories, Australia) was used at 12.5 µg/ml as a positive control for specific stimulation. PBS was included as a negative control. For all assays, the non-specific T-cell stimulator concavalin A (Sigma, USA) was included for all animals at 20 µg/ml to check cell viability.

Results were expressed as "corrected" absorbance at 450 nm. For duplicate wells, this was defined as the average $A_{450\ nm}$ of the stimulated wells (Avian or Johnin PPD or protein of the invention) minus the average $A_{450\ nm}$ of the PBS control wells. For single the protein of the invention stimulated wells, this was defined as the $A_{450\ nm}$ of the stimulated well minus the average $A_{450\ nm}$ of the PBS control wells. Differences between groups were calculated by the Mann-Whitney test. The software package InStat 2.01 (GraphPad Software Incorporated, USA) was used for statistical analysis.

Results

Cell-mediated immune responses to the protein of the invention

To investigate if recombinant protein could stimulate cell-mediated immune (CMI) responses in Neoparasec-vaccinated animals, whole blood IFN-γ assays were carried out. In the first instance, Ni^{+2}-affinity enriched recombinant protein was tested in single wells in three different concentrations (2.6 µg, 0.64 µg and 0.32 µg). Results are shown in Figure 7. There was a significant difference ($p<0.01$) between the Neoparasec-vaccinated and unvaccinated group in the IFN-γ responses to protein of the invention at all three

concentrations. Eight of the nine vaccinated animals showed IFN-γ production to all three concentrations of protein, often in a concentration-dependent manner. Animal 136 had very low IFN-γ production to protein and to Avian PPD. The low response to Avian PPD was consistent in this animal over the previous five months of testing (data not shown). None of the unvaccinated animals had notable IFN-γ responses to the protein of the invention, however, three of these animals (128, 133, 569) had comparatively large responses to Avian PPD, especially animal 569. Reactions of control animals to Avian PPD were not uncommon and appeared occasionally in various animals during testing over the previous five months (data not shown).

To demonstrate that the immunologically active component in the Ni^{+2}-affinity preparation was the protein of the invention, the protein was purified by size-exclusion chromatography and tested for its ability to stimulate IFN-γ production in whole blood. Results are shown in Figure 8. There was a significant difference ($p<0.05$) in the IFN-γ responses to 1 μg of the protein of the invention between the vaccinated and unvaccinated group, despite the low responses of animals 578 and 587 in the vaccinated group and the notable response in animal 133 in the unvaccinated group. Animal 136 was not included in the assay because of its consistently poor response to Avian and Johnin PPD. To see if the IFN-γ production to the protein of the invention was concentration-dependent, three vaccinated animals were chosen from Mob 1, along with three unvaccinated animals for testing using 5 μg of purified protein. Only three animals from each group were tested due to limited amounts of purified protein. Two animals in the vaccinated group had evident concentration-dependent responses to the antigen and the third had a slight concentration-dependent response.

EXAMPLE 5

Localisation of the protein of the invention in M. ptb

An indirect approach to investigate whether the protein of the invention is exported in its native host, *M. ptb*, was to determine if animals vaccinated with *M. ptb* strain 316F culture filtrate produced antibody to the protein.

Materials and Methods

The techniques used herein were as described in Example 4 above.

Results

Detection of the protein with serum from sheep vaccinated with M. ptb *strain 316F culture filtrate*

Serum samples from *M. ptb* culture filtrate-vaccinated sheep were used to probe individual Western blots of recombinant protein. Results are shown in Figure 9. All five vaccinated animals had antibodies to the antigen at one month post-vaccination. Antibody was not present prior to vaccination.

EXAMPLE 6

Localisation of the protein in M. ptb

A direct approach to investigate whether the protein of the invention is exported in its native host, *M. ptb*, was to determine its location in Western blots of culture filtrate fractions using rabbit antibody raised to the protein.

Materials and Methods

Preparation of the protein for immunisation

The protein of the invention was prepared for immunisation by transferring approximately 0.05 mg of Ni^{+2}-affinity enriched recombinant protein onto nitrocellulose membrane. The membrane was stained with Ponceau S and the protein band was excised, destained and air-dried. The blot was then fragmented inside a microfuge tube with the aid of a sterile scalpel blade. To this, approximately 300 µl of PBS was added and the material was further fragmented until it could pass through an 18 gauge, 1 1/2" needle. Five hundred microlitres of Freund's incomplete adjuvant (F 5506, Sigma, USA) was added and the mixture was passed through the needle several times before being injected.

Rabbit immunisation protocol

Adult New Zealand White rabbits were individually housed under standard conditions in outdoor hutches by Agricultural Services, Massey University, Palmerston North, New Zealand. Injection of prepared antigen was done subcutaneously in the mid-scapular skin fold of one rabbit. This was repeated approximately three weeks later. To check for antibody production, 5 ml of venous blood was collected from the external ear by a scalpel nick three weeks after the second injection. Serum was harvested from the clotted blood after centrifugation at 3,000 x g for 15 min. At the same time, serum was similarly collected from a naïve rabbit for use as control serum. The sera were aliquotted and stored at -20°C. Following confirmation of antibody production, the rabbit was exsanguinated and serum was harvested as above

Results

Production of rabbit antibody raised to the protein and detection of the protein in cellular fractions

To determine the cellular localisation of the native protein of the invention in *M. ptb* strain 316F, rabbit antibody was raised to recombinant protein. As shown in Figure 10, the anti-serum recognised recombinant protein on Western blots. Naïve rabbit serum did not recognise the protein. The serum was used to probe Western blots of equivalent amounts of *M. ptb* strain 316F culture filtrate. Results are shown in Figure 11. A single band of apparent molecular weight 24.2 kDa was detected in the culture filtrate, which was slightly larger than the molecular weight of 22.3 kDa calculated for the mature native protein based on its amino acid composition. This band was also present in greater intensity in both the soluble and insoluble cell preparations. Several other weak bands of higher molecular weight were produced in the soluble and insoluble cell fractions with the naïve and recombinant protein-immunised rabbit sera. Comparison to Ni^{+}-affinity chromatography prepared recombinant protein produced from *M. smegmatis*, previously shown to migrate with an apparent molecular weight of 23 kDa, confirmed native protein migrated slightly slower than recombinant protein.

EXAMPLE 7

To determine if sequences related to the gene encoding the protein of the invention existed in other mycobacterial species, nucleic acid database searches, PCR and Southern blot analyses were carried out.

Materials and Methods

PCR amplification of the protein-encoding ORF from genomic DNA

PCR amplifications were carried out on mycobacterial genomic DNA using the primer pairs lpp27-fBam and lpp27-rKpn. The reactions were carried out in 20 µl using *Taq* DNA polymerase. Template DNA consisted of 2 µl of a 1:50 dilution of purified DNA or, alternatively, single bacterial colonies were added directly to the PCR mixture.

Southern blotting and hybridisations

Approximately 1 µg of restriction endonuclease-digested genomic DNA fragments were electrophoresed in 0.7% agarose gels and transferred to nylon membranes (Biodyne B, Gelman, Pall Corporation, USA) by capillary transfer using standard procedures (Sambrook *et al.*, 1989). The DNA was fixed to the membrane by exposure to UV light for 4 min using a Bio-Rad Gel Doc 2000 transilluminator (Bio-Rad, USA). Fixed membranes were prehybridised in heat-sealed plastic bags with DIG Easy Hyb buffer (Roche Molecular Biochemicals, Germany) at 42°C for 2 h with constant shaking in a Hot Shaker water bath (Bellco Biotechnology, NJ, USA). Hybridisation was done in the same buffer at 2.5 ml/100 cm^2 with 25-50 ng/ml of denatured DIG-labeled probe DNA at 40°C for 18 h. The hybridised membranes were washed 2 x 5 min in 2 x SSC, containing 0.1% (w/v) SDS at room temperature, followed by 2 x 5 min washes in 0.7 x SSC containing 0.1% (w/v) SDS at 68°C with constant shaking. Immunodetection of hybridised probe was achieved using the DIG system (Roche Molecular Biochemicals, Germany). Briefly, washed membranes were incubated in 1 ml/cm^2 blocking solution (Roche Molecular Biochemicals, Germany) for 60 min at room temperature with shaking. The blocking

solution volume was reduced to 20 ml/100cm^2 and anti-DIG antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Germany) was added to a final dilution of 1:10,000 according to the manufacturer's recommendations. The blots were developed by chemiluminescence with CSPD or CPD-Star substrate (NEN, MA, USA). The developed blots were exposed to radiographic film (BioMax MR, Kodak, USA) for 5 min to 18 h, depending on signal intensity, in the presence of a single intensifying screen (Kodak Lanex Regular, Kodak, USA). Film was developed in an automated processor (Kodak RP X-OMAT Processor Model M6B).

DNA probe preparation

All probes were labeled by the incorporation of DIG-labeled dUTP (DIG-11-dUTP, Roche Molecular Biochemicals, Germany) during PCR. DIG-11-dUTP (1 573 152, Roche Molecular Biochemicals, Germany) was added to a final concentration of 20 μM in a reaction volume of 50 μl and dTTP was adjusted to a final concentration of 80 μM. All other deoxynucleoside triphosphates were added to 100 μM. To estimate the purity and yield of DIG-labeled product, approximately 2 μl of the reaction was electrophoresed in agarose gels alongside a mass ladder for quantitation (10068-013 Low Mass DNA Ladder, Life Technologies Inc., USA). Due to the presence of DIG, the PCR products routinely appeared larger than unlabeled products. For quantitation of DIG-incorporation in probes, side-by-side filter spot tests, ranging from 0.01 pg to 10 pg, were carried out as per the manufacturer's recommendations. Labeled PCR products were stored at -20°C until used for hybridisation.

Results

PCR amplification of the gene encoding the protein from Mycobacterium species and strains

To determine if the gene encoding the protein of the invention was present in other *M. ptb* strains, several isolates representing five IS*900* RFLP types (Collins *et al.*, 1990) were used in PCR analyses. The results are shown in Figure 12. The expected 725 base pair product was amplified from all 13 isolates.

To investigate the species distribution of the gene encoding the protein of the invention within the genus *Mycobacterium*, PCR reactions were carried out with a limited number of mycobacterial species, including two strains of *M. ptb* and 11 strains from the *M. tuberculosis* complex. Primers lpp27-fBam and lpp27-rKpn were used. Amplification of the genus-specific 16S rRNA gene was carried out in parallel for all DNA species and served as a positive control for the reactions. Annealing temperatures of 55°C and 62°C were employed (the calculated Tm of the primers was 66°C in 50 mM NaCl). Results are shown in Figure 13. PCR products of the expected size (725 base pairs) were amplified from both of the *M. ptb* isolates as well as *M. scrofulaceum* and *M. intracellularae* using both annealing temperatures. The only other product of the expected size for the gene of interest was produced at 55°C, in the *M. tuberculosis* complex strains (Figure 13b; lanes 12 - 22), however this product was very weak and was not produced at 62°C. A major band at 250 base pairs was produced with *M. terrae* at 55°C, however this product was not produced at 62°C. Various weak products were amplified from several of the other species, but none of these were produced with consistency at both annealing temperatures.

Southern blot detection of the gene of interest in Mycobacterium species and strains

Southern blot analyses were performed with *Bam* HI-digested genomic DNAs from 13 *Mycobacterium* spp. The ORF encoding the protein of the invention was used as probe and was labeled by incorporation of DIG-dUTP during PCR using the primers lpp27-fBam and lpp27-rKpn. Figure 14 shows the probe hybridised strongly to a single band, approximately 2,200 base pairs in length from *M. ptb* 316F and a slightly larger band of 2,300 base pairs from *M. ptb* ATCC 53950. The probe also hybridised weakly to a 1,000 base pair band from *M. intracellularae*. Upon overnight exposure (data not shown), a weak band at approximately 10,000 base pairs was present in *M. marinum*, *M. terrae*, *M. phlei* and *M. kansasii*. Hybridisation was not detected with *M. bovis*, *M. tuberculosis*, or *M. bovis* BCG.

It will be appreciated that the above description is provided by way of example only and that the variations in both the materials and the techniques used which are known to those persons skilled in the art are contemplated.

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Figure 1.

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Figure 2.

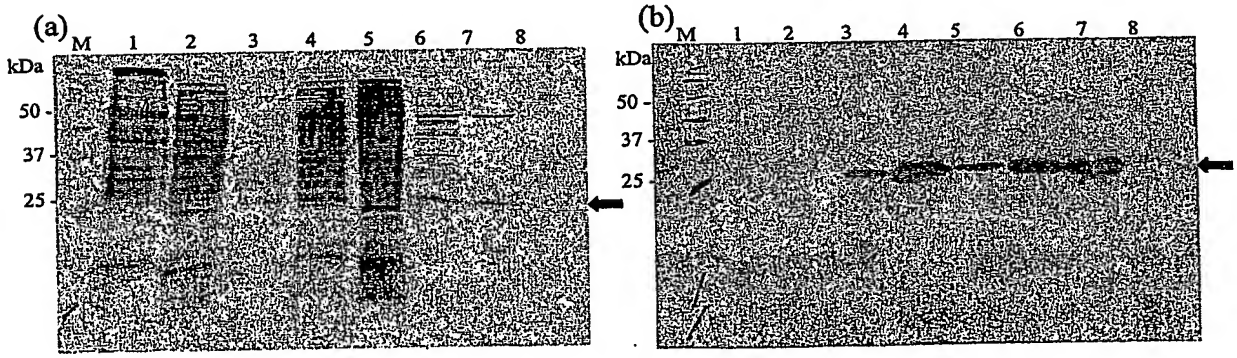


Figure 3.

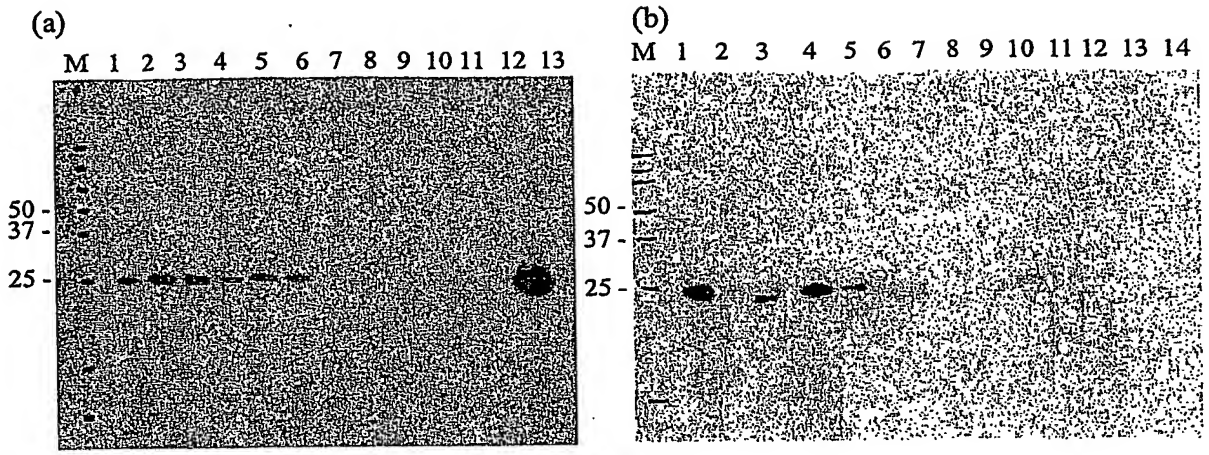


Figure 4.

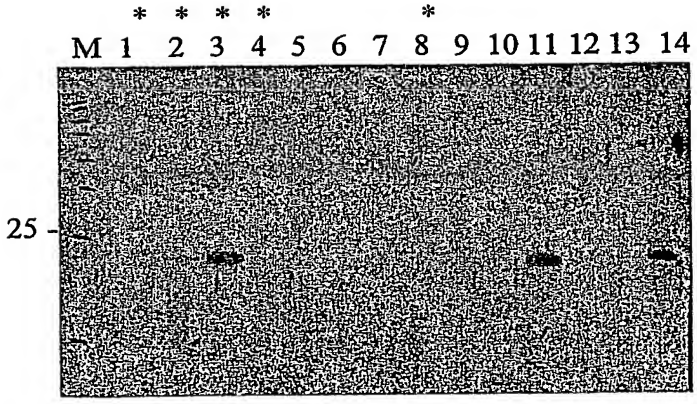


Figure 5.

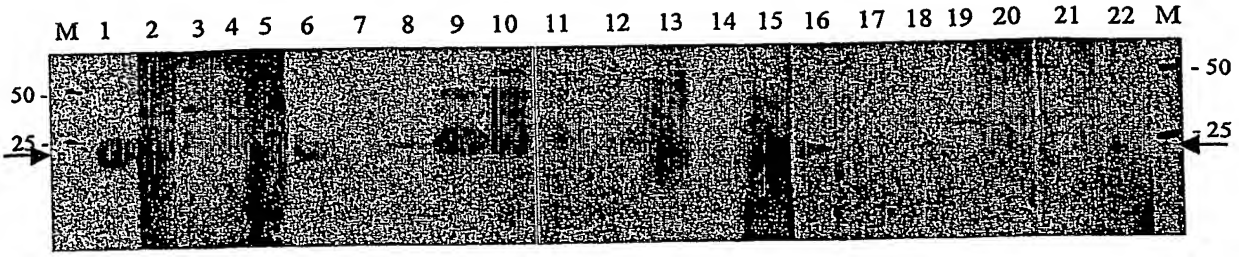
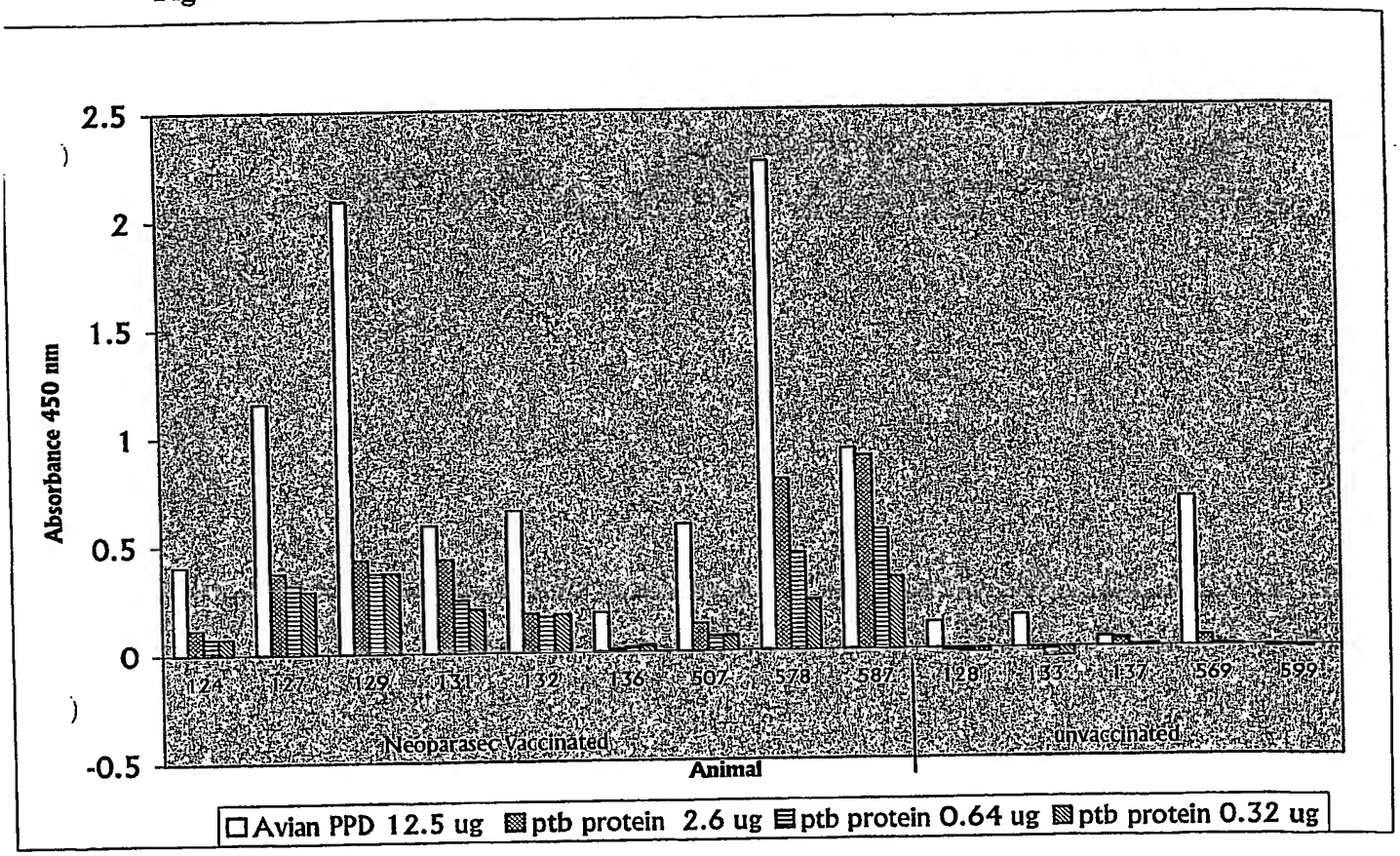


Figure 6.



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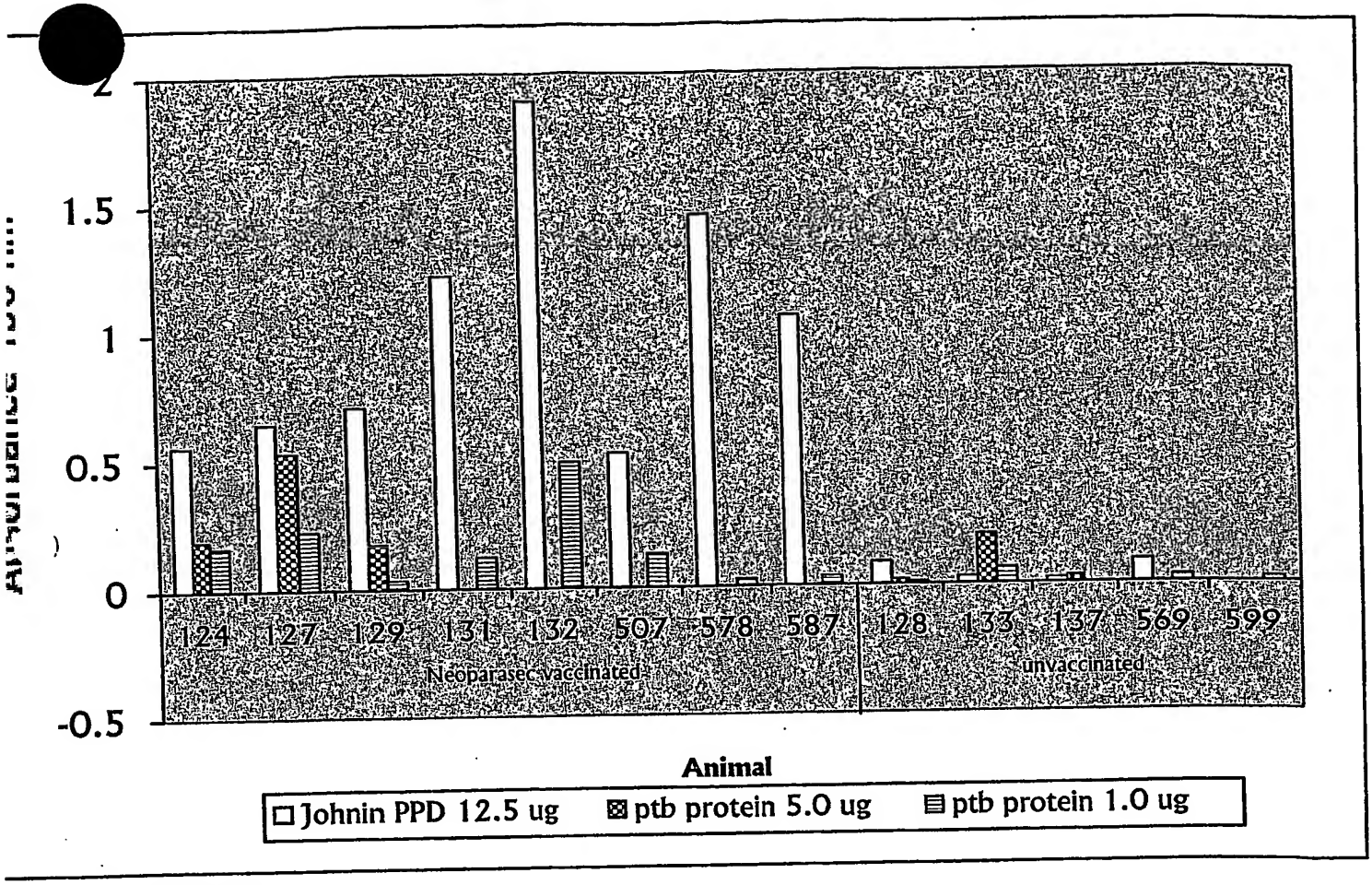


Figure 8.

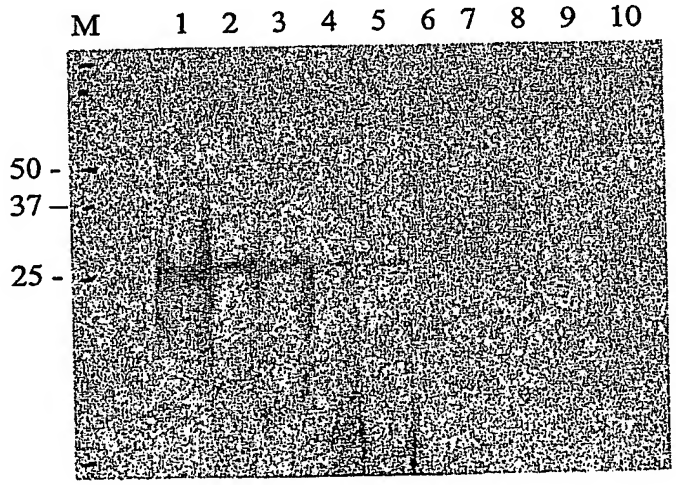


Figure 9.

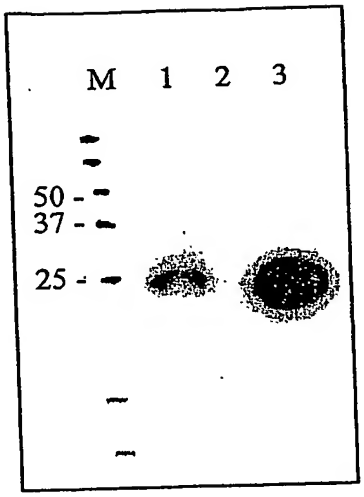
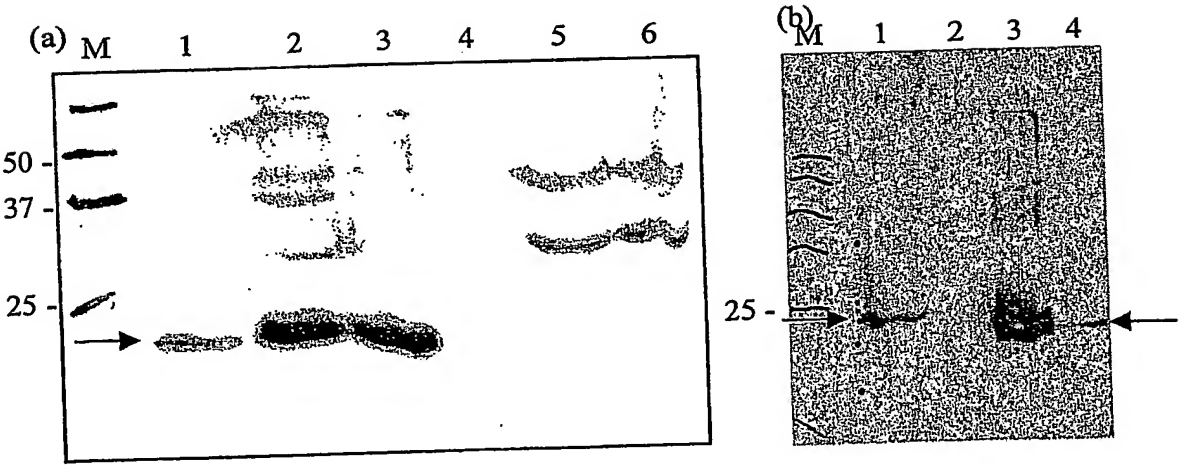


Figure 10.



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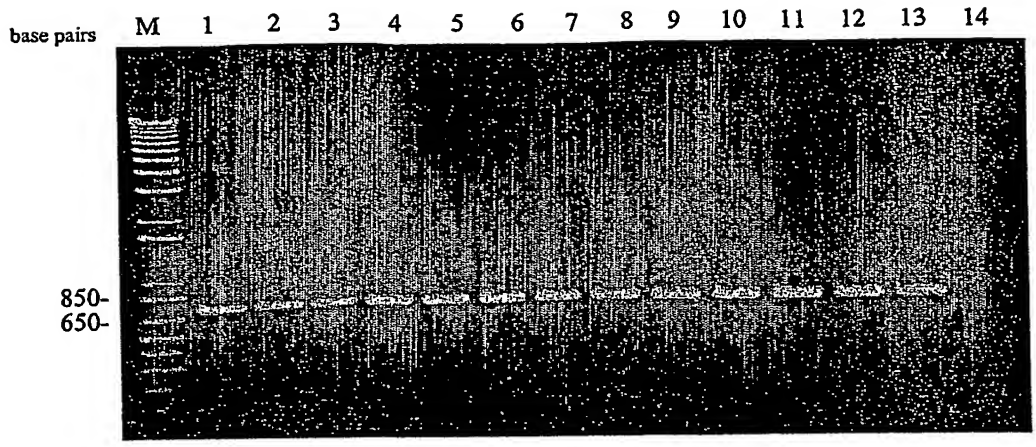

Figure 12.

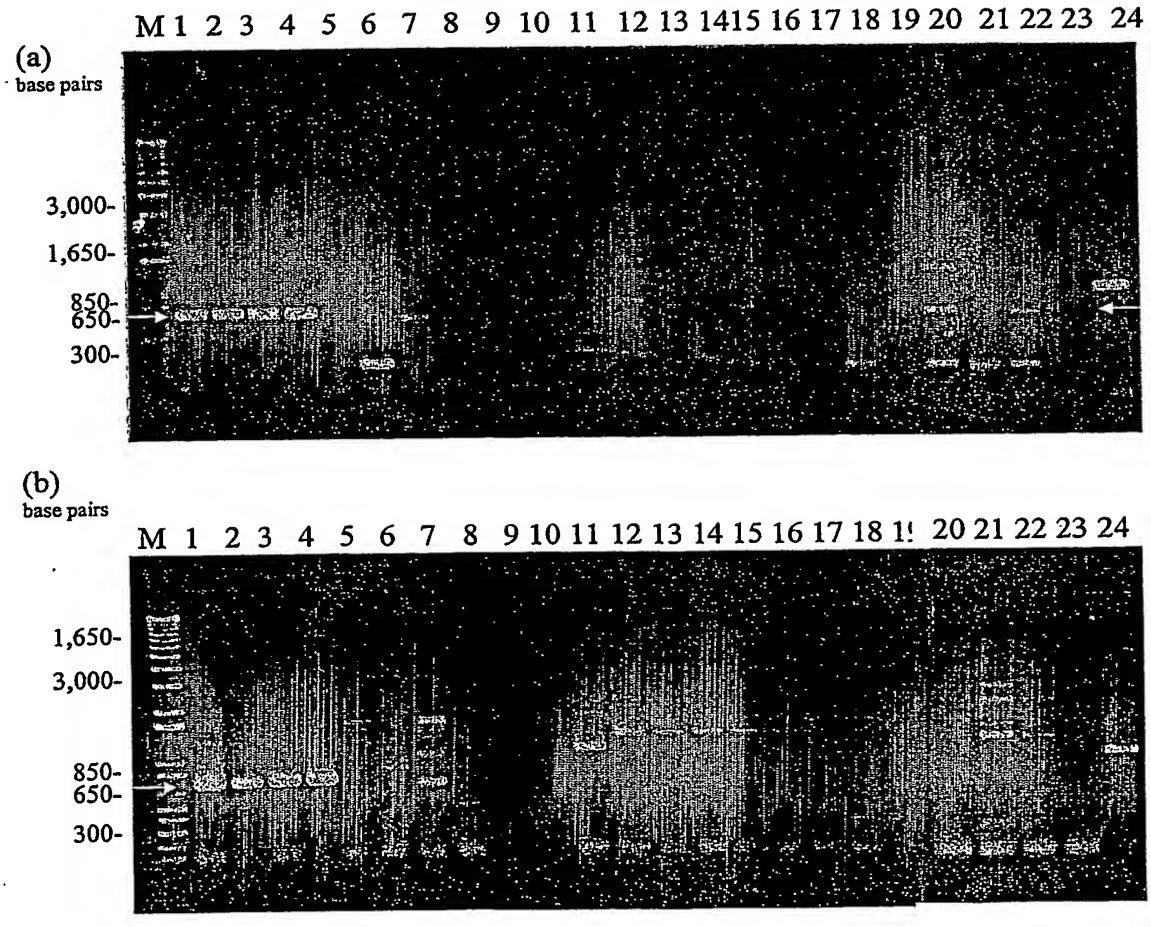

Figure 13.

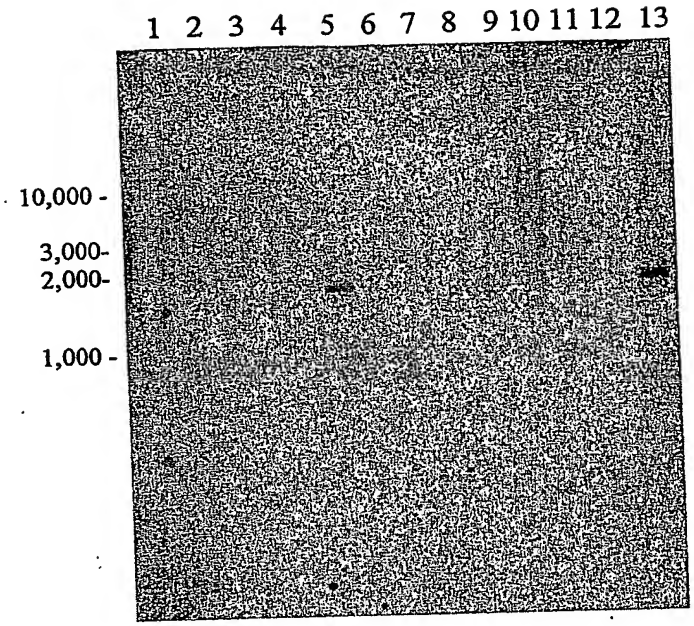

Figure 14.

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